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THE MEASUREMENT OF DEXTROSE IN STANDARD SOLUTIONS WITH DREYWOOD'S ANTHRONE REAGENT AND THE KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER

(A Statistical Evaluation and a Simple Microprocedure)

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SUMMARY PAGE

THE PROBLEM

To adapt the anthrone-hexose reaction for use with smaller amounts of materials than have hitherto been considered necessary for quantitative determinations of carbohydrates.

FINDINGS

A macro-procedure was developed which demonstrated extreme sensitivity and accuracy, such as would make it suitable for use in a clinical laboratory and other places where precision may be required. Its advantages and limitations are tabulated.

APPLICATION

The findings of this report will be useful to clinical laboratory workers concerned with accurate measurement of dextrose in extremely small amounts of materials.

ABSTRACT

An experiment was performed using a macro-procedure for the anthrone-hexose reaction with standard solutions of dextrose and a Klett-Summerson photoelectric colorimeter in order to reveal significant sources of error and variability and to assess the upper limits of accuracy of this reaction with reference to its suitability for the clinical laboratory and other places where precision may be required. Sensitivity and accuracy were found sufficient to permit the introduction of additional errors arising from the use of biological fluids without necessarily removing the anthrone-method from a position of advantage in competition with other procedures. Slow fading of the anthrone-carbohydrate complex occurred after 8 hours at 24°C., but this fading was undetectable if specimens were read as soon as they cooled. When special reaction tubes designed to conserve heat were used in a warm room, the anthrone-hexose reaction obeyed the Bouguer-Beer law for dextrose in quantities of 3.75 to 20 gamma in 0.75 ml. of original solution. Two supplementary methods of controlling temperature in order to obtain satisfactory color development are considered, and the effects of their improper use are pointed out. A simple procedure is proposed for using Dreywood's anthrone reagent to measure "sugar" in 20 cu. mm. of blood collected in a Sahli pipette.

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INTRODUCTION

The primary objective of this pilot study was to adapt the anthrone-hexose reaction (1) for use with smaller amounts of materials than have hitherto been considered necessary for quantitative determinations of carbohydrate. It was hoped that a simple procedure could be devised which might ultimately be standardized for measurements upon blood from small animals or collected from a fingertip with a Sahli pipette containing 20 cu. mm., and for other purposes as well. This need explains the substitution of microtubes for the usual Klett tubes; but since the inside diameter of both tubes is the same, the applicability of our findings is not necessarily limited to the microtubes we happened to use — except, perhaps, with regard to the variability of measurement attributable to the tubes themselves.

Part of the investigation undertook statistical evaluations of some of the more fundamental sources of error likely to be associated both with the reaction itself and with a reasonably typical macro-procedure utilizing it. Because one function of the macro- determinations made in this connection was to provide an appropriate frame of reference for the results of the micro-method, the same operations and equipment were used in both cases whenever this could be done without unnecessary sacrifice of accuracy or information.

Although an entirely satisfactory chemical explanation for the behavior of Dreywood's anthrone reagent in the presence of carbohydrate is yet to be offered (1,5), this reaction has been sufficiently investigated to establish its usefulness in the clinical laboratory as well as elsewhere (7,9). Requiring no complicated reagents or elaborate or unusual equipment, the procedure is extremely simple and almost effortless. The Bouguer-Beer law is obeyed at least between the values of 30 and 100 micrograms for a mixture of galactose and mannose (6), and from 4 to 600 micrograms for dextrose or glucose (4; this report). Further study may extend these limits in both directions. Measurements of blood-sugar with anthrone and by more conventional means agree closely enough to justify the easier technique for most purposes (2).

Objections to the use of anthrone are not serious and concern mainly:

(1) the fact that it probably measures total carbohydrate rather than glucose specifically,

(2) the care needed in the use and disposal of solutions of concentrated sulphuric acid,

(3) minor color-changes in both the anthrone reagent and the specimen prepared for the photometer when they stand at room-temperature,

(4) impairment of accuracy by low or markedly changing temperatures in the laboratory, and

(5) the fact that the presence of two colors in all solutions to be read except the blanks renders visual colorimetry unreliable.

The chief obstacle to the micro-determination of carbohydrate with Dreywood's anthrone reagent seems to be the difficulty of controlling heating and the dissipation of heat in satisfactory amounts and patterns when small volumes are employed (7). The simple device of relying upon aqueous dilution of 95% sulphuric acid containing anthrone, and subsequent cooling in room-air, has thus far been considered suitable only for work on a macro scale (4). However, an obvious alternative to experimenting with the less convenient methods of regulating

temperature used in some experiments (3,6) is to perform the dilution in a spherical container, which offers the least surface for its capacity. Such a vessel is also well suited for rapid and thorough mixing. If necessary, a standard solution or one derived from the material to be measured could be preheated in it to an empirically determined optimal temperature. Cooling could be retarded by surrounding the container with glass wool during color-development. We have found that in a moderately warm room, neither preheating nor insulation is necessary.

Statistical Evaluations of Certain Sources of Error Associated with a Macro-Procedure

Method. Four solutions of dextrose were prepared from a stock standard in such a way that 5 ml. of each solution respectively contained 25, 50, 100, and 200 gamma (1 gamma = 1 microgram) of dextrose on the first of 2 days, and 25, 100, 150, and 200 gamma on the second day. These values also provided a reference-line for our micro-procedure for measuring "sugar" with 20 cubic mm. of blood. In duplicate series representing the blank and the four concentrations of sugar available at one time, 5 ml. of appropriate sugar-solution or distilled water were placed in 25 x 150 mm. heat-resistant test tubes. Ten ml. of day-old anthrone reagent (0.2% in 95% sulfuric acid) were then added in such a way as to cause layering rather than irregular and slow mixing (7). A 10 ml. volumetric pipette was used for this step the first day, and a 50 ml. burette on the next. Immediately after the addition of anthrone, the layered solutions were mixed abruptly by swirling, and the tube was allowed to cool to room-temperature without interference.

When one of the blanks was cool, 2 ml. portions were transferred from it into 6 permanently numbered Klett microtubes (A. H. Thomas, Catalogue No. 3788-D5). This was repeated with the second blank. The outside surfaces of the microtubes were cleaned at room-temperature and wiped clear with gauze. Each of the three observers then made two consecutive series of photometer readings for the twelve microtubes, taken in the same order both times and with preceding data concealed from view. Between every two readings, the zero adjustment of the photometer was checked against a clean microtube of distilled water. The observers were instructed to avoid errors caused by parallax.

Shortly before the work with one pair of parent-solutions was complete, the next two were subdivided, and so on. In the course of one day covering more than eight hours, sixty microtubes were used and 72 readings were obtained for each pair of large tubes. None of the solutions was refrigerated. The blanks were read first on both days, but on the first day the other parent-solutions were taken in the order of ascending concentrations of dextrose, and on the second day in the order of descending concentrations. Also, on the second day, the sequence of microtubes was changed from 1, 2, 3, 4, 5, 6, to 3, 2, 1, 6, 5, 4, etc. Except for the blanks and 100 microgram standards, no set of 12 microtubes was used for the same concentration of sugar on both days.

The maximal spectral absorption of the anthrone-carbohydrate complex is at approximately 625 millimicrons and remains constant despite slow fading (7). As might be expected, we found that a filter with maximal transmittance in this region affords greater selectivity of measurement than others. (See also (4).) Because of too great optical density, however, not all filters which meet this specification are suitable for the Klett-Summerson photometer. We used the standard 100-watt light-source and a combination of two filters which had, together, very little transmittance below 590 millimicrons (cf. (6)) and a calculated maximal transmittance at about 630 millimicrons. They were:

Corning glass filter No. 2412 (red)
3.03 mm. thick. Melt 784
Corning glass filter No. 3961 (green)
2.57 mm. thick. Melt 905

Results
1. **Fading of the anthrone-carbohydrate complex at room -temperature (24° C.).**

This was not of primary concern in the present study, and is ordinarily rendered insignificant by prompt reading of specimens after they have cooled. For further information, see references (2) and (7).
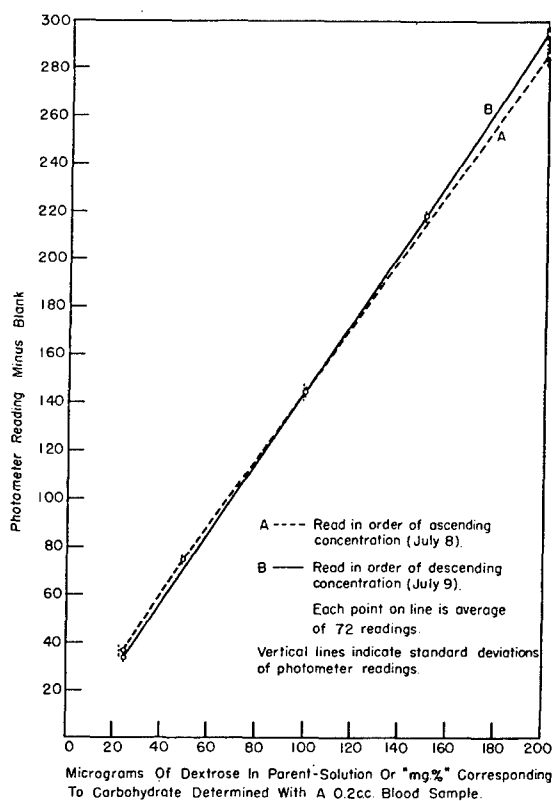
Figure 1.—Averages and standard deviations of determinations for each concentration of dextrose on separate days.

Our data reveal fading and something of its extent. Fig. 1 shows photometer-readings-minus-blank (ordinate) plotted against micrograms of dextrose in the parent-solutions from which the Klett microtubes were filled (abscissa). The base-line may also be read as "mg.%" corresponding to sugars determined with 0.2 cc. samples of blood, as they are analyzed in our laboratory. Each point is as average of 72 values. Standard deviations for the readings are represented by vertical lines.

The dotted line (A) was obtained from microtubes read in the order of ascending concentrations of dextrose. Consequently, there was an interval of about 8 hours between the preparation and reading of the 200 gamma specimens in this series. For the solid line (B), the reverse order was followed, and the 25 gamma specimens were read last. Means for the blanks were identical on both days — a fact which lends weight to the following considerations by

making it unnecessary to take into account optical differences in the anthrone reagents themselves.

The differences between the two lines are uniformly consistent with their interpretation as the result of slow fading or decrease in optical density. Moreover, it happened that the 100 gamma specimens were read after a lapse of the same amount of time each day. The intersection of the lines at this concentration of sugar is what we should expect if color-loss had proceeded at generally similar rates on both occasions.
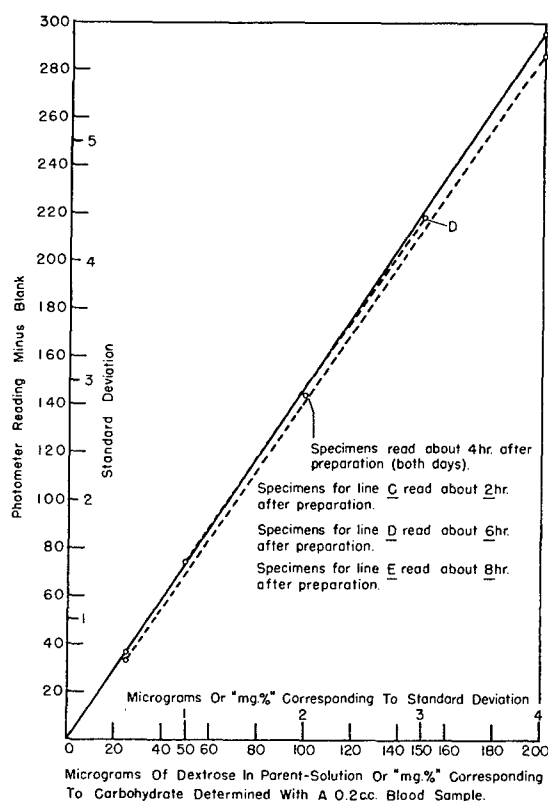
Figure 2.—Determinations for specimens read at similar intervals after preparation.

A line (C, in Fig. 2) drawn through the points representing the 25 gamma specimens of series A and the 200 gamma specimens of series B passes almost exactly through the origin, as would be required if the two points are accurate and if the blanks and sugar-solutions were reasonably free from error. Line C gives smaller discrepancies than either A or B when the sugar and photometer value for any one point are used as "knowns" to calculate the sugar correspond-

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ing to another photometer value. Finally, the same line conforms closely to one obtained by a micro-method in which all specimens were read within a few minutes after they had cooled (Part II).

The difference between the means for the readings of the 25 gamma specimens on the two days is significant, the level of signifiance being somewhat better than 3%.* The means for the 200 gamma specimens differ with a very high degree of significance. In this case, the level of significance is better than 0.1%. Adopting line C in Fig. 2 as the most valid, we find that changes attributable to fading during 8 hours at 24°C. are approximately equivalent to 2 mg.% (8%) for 25 gamma and 6.5 mg.% (3.3%) for 200 gamma.

In Fig. 2, points representing means for specimens read after similar intervals following preparation of the parent-solutions are connected by straight lines. Only one point is entered for all the 100 gamma tubes. This illustration shows both a progressive fading with lapse of time, and greater declines in density for higher concentrations of sugar. The last observation supplements evidence obtained by Morris (4) and ourselves (Part II) that color-development in the presence of larger amounts of sugar is more adversely affected by improper control of temperature during the anthrone-carbohydrate reaction.

Table I

Means and Standard Deviations of Photometer Readings for each Parent-Solution

Parent-Solution	First Day		Second Day	
	A,	B,	A₂	B₂
Conc. of Dext.				
Blank	46.7∓1.54	45.4∓1.40*	46.2∓1.58	46.1∓1.65
25 γ	81.8∓3.00	82.2∓1.00	79.8∓1.09	79.6∓0.83
50 γ	121.0∓1.27	119.7∓1.67*		
100 γ	192.9∓2.99	187.3∓2.27*	189.6∓2.30	191.3∓2.04
150 γ			264.0∓2.74	264.3∓1.27
200 γ	335.2∓3.36	328.9∓2.45*	341.3∓2.83	341.9∓1.72

Anthrone reagent measured with 10 ml. vol. pipette.   Anthrone reagent measured with 50 ml. burette.

*Asterisk indicates that the difference between the means of the duplicate parent-solutions is statistically significant. Multiply either ordinate of Fig. 2 by 0.68 to convert it to micrograms or "mg.%".

2. Agreement between duplicate parent-solutions.
Table I gives the means and

standard deviations of the photometer readings for each parent-solution on the two days. An asterisk indicates that the difference between the means of duplicate preparations is statistically significant. The fact that the differences are significant for all but one concentration of dextrose on the first day, but in no instance on the second, shows that inaccuracies of measurement were more appreciable when a 10 ml. volumetric pipette was used to deliver the anthrone reagent into the parent-tubes. Neither such a pipette nor an ordinary burette is designed for precision with a solution more viscous than water. A detectable random error was apparently introduced by allowing the pipette to drain for slightly different lengths of time against the inner wall of a parent-tube. On the other hand, when effort was made to deliver the anthrone reagent from a 50 ml. burette at the same rate each time, the error which undoubtedly occurred seems more likely to have been fairly constant. A constant error large enough to be exposed graphically could be corrected in subsequent calculations if desired. The standard deviations and means in Table I may be converted to micrograms or "mg.%" by referring to line C, the appropriate ordinate, and the inner abscissa of Fig. 2, or by multiplying them by 0.68. The preceding observations are presented for two reasons: (a) to indicate the smallness of differences in concentration of sugar which can be revealed by using anthrone and a photoelectric photometer, and (b) to show the need for particular care to achieve accurate reproduction of the volume of the anthrone reagent for maximal precision in measuring carbohydrate.

3. The agreement of each observer with himself.
The three participants in this study were experienced in either or both psychophysical and clinical laboratory procedures. Observers 1 and 2 had frequently used the Klett-Summerson photometer before.

Table II lists, according to concentration of dextrose in the parent-solution, the means and standard deviations of the photo-

Dextrose	Statistic	Observer 1			Observer 2			Observer 3			All Observers
		Day 1	Day 2	Both	Day 1	Day 2	Both	Day 1	Day 2	Both	Either or Both
Blank	M	46.3	46.1	46.2	45.5	45.6	45.6	46.5	46.8	46.7	46.1
	σx	1.45	1.44	1.45	1.16	1.54	1.34	1.77	1.63	1.55	1.60
25 γ	M	83.0	79.4		81.6	80.4		81.2	79.4		
	σx	2.41	0.46		1.70	0.32		2.12	0.75		
50 γ	M	151.2			120.6			119.1			120.4
	σx	1.20			1.14			1.66			1.59
100 γ	M	191.9	191.7	191.8	190.8	190.7	190.7	187.7	189.0	188.3	190.3
	σx	2.99	2.17	2.66	4.09	2.32	3.32	2.95	1.50	2.51	3.16
150 γ	M		265.5		263.3			263.5			264.1
	σx		1.96		1.75			2.03			2.30
200 γ	M	331.3	342.2		334.3	341.4		330.5	341.3		
	σx	3.26	2.37		4.68	1.87		3.98	2.54		

In these analyses, readings for 12 microtubes from two parent-solutions representing each concentration of dextrose, or the blank, were grouped together.

M=Mean

σx=Standard deviation of photometer-readings

meter readings of the observers singly and, where reasonable, in combination. In the calculations for this Table, readings for the twelve microtubes derived from each pair of parent-solutions were grouped together. This follows a common practice of preparing duplicates and combining the determinations from them. Variability obviously should be increased insofar as duplicates are not measurably identical, and will change with both the sensitivity of the instrument and the fineness of its scale.

Again, standard deviations and means are convertible to micrograms of dextrose or "mg.%" with the aid of Fig. 2 or by multiplying them by 0.68.

Table III

Amount of Dextrose and Percentage of Error Corresponding to the Maximal Standard. Deviation of Photometer Readings for each Concentration of Dextrose.

Conc. of Dextrose	Maximal Stand. Dev.		Equiv. in σ or "mg.%"		Percentage of Error	
	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
Blank	1.77	1.63	1.2	1.1	4.8*	4.4*
25γ	2.41	0.75	1.6	0.5	6.4**	2.0**
50γ	1.66		1.1		2.2	
100γ	4.09	2.32	2.8	1.6	2.8	1.6
150γ		2.30		1.6		1.1
200γ	4.68	2.54	3.2	1.7	1.6	0.9

* Expressed in terms of 25γ which would be the value most affected. An error in the blank will summate algebraically with the errors of other measurements. Its presence is suspected, though not proved, when the line representing photometer-readings-minus-blank for standard sugar solutions fails to pass through the origin.

** 11.2% and 6.4%, if deviations for blank and 25γ are added.

In preference for a conservative approach and because of demonstrable differences between duplicate parent-solutions on the first day only, the largest standard deviations in Table II have been arranged in Table III according to both the day and the amount of dextrose. With them are given their equivalents in micrograms (or "mg.%") and the percentages of error with respect to appropriate concentrations of sugar. All but the standard deviation for 150 gamma represent individual observers. It will be recalled that 68% of observations can be expected to fall within plus or minus 1σx from the mean. Variability of the order shown in Table III, especially for Day 2, is small enough to justify the conclusion that anthrone is capable of measuring carbohydrate with sufficient accuracy for clinical and for many experimental purposes.

Table IV

Analysis of Variance for Pairs of Parent-solutions* Read by Different Observers, and with Two Series of Mean Readings for Each Pair

Source of Variation	Degrees of Freedom	Mean Square	F
Between observers	2	6.415	<1.00
Between pairs of parent-solutions read by same observer	9	68466.31	
Total between pairs of parent-solutions	11		
Between 4 series of mean readings for each conc. of dextrose, both days	3	18.767	1.72
Interaction: 4 series of mean readings x observers	6	1.093	<1.00
Interaction: All solutions x four series of mean readings	27	9.670	
Total within observers	36		
Total variance	47		

*Each datum used in computation was a mean of 12 readings from 2 parent-solutions representing one concentration of dextrose or the blank.

Analysis of variance (Table IV) shows that the variability of each observer with respect to the averages of twelve readings for each concentration of dextrose is attributable to chance. Random variability is indicated by the fact that none of the F-values in this table is significant. Application of Bartlett's test of the homogeneity of variance reveals that the variances are not heterogeneous. It may also be concluded from this table that variability is not significantly affected by concentration of sugar.

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4. **Agreement between observers.** From Table II, it may be seen that the standard deviations for the three observers considered separately and together are similar. Through analysis of variance (Table IV), it is found that the variability between observers is attributable to chance. The practical implication of these statements is that reasonably careful observers may be used interchangeably without serious impairment of accuracy.

5. **The possibility that certain microtubes may cause significant errors.** For utmost accuracy, it is desirable to know whether certain microtubes without apparent defects cause photometer-readings to differ consistently from those obtained with the majority of tubes. Such information requires more extensive data than ours for each microtube. However, it is possible to estimate an error which is likely to be significant and which could justify rejection of any tube shown to be responsible for it. If it is assumed that 99.7% of all photometer readings in a particular group fall within plus or minus $3\sigma_X$ from their mean, reference to the maximal standard deviations given in Table III for the second day (when the members of each pair of parent-solutions can be considered equivalent) will reveal that these limits are plus or minus 4.89 photometer units (3.3 gamma) for the blank and plus or minus 2.25 units (1.5 gamma) for 25 gamma of dextrose. The figures in parentheses represent deviations of about 13.2% and 6% from 25 gamma. Among the sixty microtubes, eleven showed, on one day, average deviations of more than $1\sigma_X$ from the means for their respective groups of six filled from the same parent-tube; and average deviations of the same sign on the other day (6 greater and 5 less than the means for their groups). In three cases, the smaller of the two average deviations also appoximated or exceeded σ_X. From blanks to 100 gamma inclusive, the differences for these eleven tubes averaged 1.7 photometer units (or 1.9 units if blanks are excluded), which is larger than 3σ for 25 gamma of dextrose on Day 2.

It therefore appears within reason that an appreciable minority of microtubes may differ from the average (in inner diameter?) enough to affect significantly the photometer readings for blanks and low concentrations of sugar. However, many clinical and experimental requirements are not so rigorous as to justify the considerable effort needed to detect these tubes photometrically.

6. **Estimation of the smallest number of specimens required for average determinations to fall within specified limits of error for each concentration of dextrose.** These estimates arose with pure solutions of dextrose, and under the assumption that the members of each pair of parent-solutions on the second day are equivalent. None of this is, of course, true with actual measurements of "blood-sugar" in the clinic. It would seem that the practice of using duplicates indiscriminately is a bit unsafe if a "true" mean is desired with any certainty.

The estimates were obtained by converting a percentage of error (5% or 10%) expressed in terms of micrograms or "mg.%" for each concentration of dextrose, into units on the photometer-scale and then by letting this value equal three times the standard error of the mean (σ_M) of all photometer-readings for that particular solution of sugar (Day 2). The number of replications*-plus-one (N) was derived from the following formula, in which σ_X is the standard deviation for photometer-readings (Table III, Day 2):

$$\sqrt{N-1} = \frac{\sigma_X}{\sigma_M}$$

It turns out that under the optimal conditions for accuracy on the second day of this experiment, reasonable expectation of an error of 5% or less from a "true" mean photometer-reading would necessitate using three each of both the blank and the 25 gamma preparation, and two each of the rest. For an error of 10% or less, duplicates would suffice for all. These figures do not take into account the important fact that errors with the blank and with a solution of sugar can summate in computation.

*The word "replication" does not include the original.

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Discussion.

The measurement of dextrose with anthrone and the Klett-Summerson photometer was studied in our Laboratory because we have previously used both the method and the instrument. Our future program, however, does not justify pursuit of this subject beyond the work being described in this report. The approach outlined and begun here is based upon the premise that the first step in the evaluation of a laboratory procedure which might become widely used should be a detailed analysis, under rigorously controlled conditions, of both the separate and the combined roles of technician, instrument, and substance-to-be-measured. Only by thus giving systematic attention to these three essential links in the chain between raw material and laboratory data is it possible to expose, weigh, and correct significant sources of error and variability which might otherwise become inseparably confused. Moreover, by revealing the best which might be expected under clearly specified conditions, such an evaluation should help an investigator to decide quickly whether a method deserves further consideration for his own particular needs. The last point constitutes the basic relationship between the two parts of this report.

It should be emphasized that, for the reasons given, our observations were arbitrarily limited to solutions of dextrose uncomplicated by other substances (6) which may be capable of affecting photometric readings for the hexose-anthrone reaction. Concentration of dextrose is a common standard of reference for measuring blood-sugar, and our exclusive concern with it is especially appropriate as long as it promises to serve the same purpose for determinations made with anthrone — regardless of whatever qualifications or corrections might be deemed necessary in order to arrive at final values with blood.

PART II

A Simple Microcolorimetric Procedure

Special Equipment.

1. **Reaction tubes.** As was expected, numerous trials of a microprocedure with tests tubes of ordinary shape and ranging in size from Kahn tubes (10 x 75 mm.) upward showed all of them to be unsatisfactory. We next tried the lower portions of Folin-Wu tubes cut off at the middle of the constrictions. These gave results which were much better but still not good enough. Since the bulbs of Folin-Wu tubes are far from round and are quite unequal in size, shape and thickness, we obtained reaction-tubes made to our specifications from the Macalaster Bicknell Co. (New Haven, Conn.). They required no modification and are described as follows (Fig. 3, left):
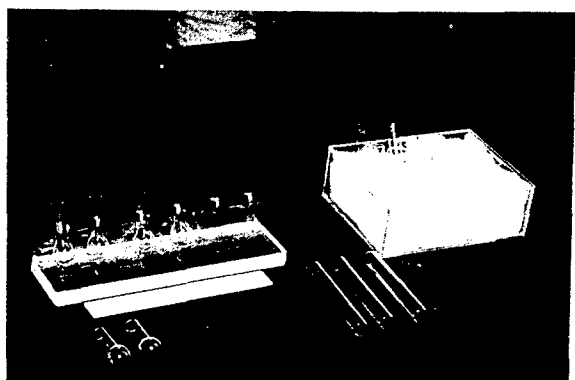

Figure 3. Some equipment used in the micro-procedure.
Reaction tubes and holder for reaction tubes at left.
Microtubes for Klett-Summerson photoelectric colorimeter in center.
Insulating box partly filled with reaction tubes on right.

The glass is heat- and acid-resistant. Size, shape and thickness of the bulb are reasonably uniform from one tube to another. The overall height of the tube is about 5 cm., the length of the stem about 3 cm., and the inner diameters of stem and bulb respectively about 1 and 1.7 cm. The bulb holds 2.25 ml. with just enough additional space to facilitate mixing of its contents by brief swirling. The stem is long enough for easy manipulations but short enough for the insertion of tips of pipettes and

burettes down to the beginning of the bulb. The transition from the stem to bulb does not hinder layering of the anthrone reagent beneath the sugar-solution while the former is being measured from a burette.

2. **Holder for reaction tubes.** (Fig. 3, left) The holder consists of a lucite base and an upright portion of thin sheet metal cut and bent to form clips along its upper edge. The upright is bent toward the base, and the clips in the opposite direction, in order to prevent the bulbs from touching metal while cooling.

3. **Insulating box.** (Fig. 3, right) This is a plastic box filled with glass wool teased apart to receive 12 reaction tubes. Holes through the cover are large enough to admit the bulbs but prevent the tubes from spilling.

4. **The special filter** and the

5. **Microtubes for the Klett-Summerson photoelectric colorimeter** were also used in Part I.

A total of 10 series of duplicate specimens representing blanks and hypothetical blood-sugars of 25, 50, 150, and 200 mg.% for determinations made upon 20 lambda of blood were prepared and read by two persons working independently. The corresponding amounts of dextrose actually present were 3.75, 7.5, 15.0, 22.5 and 30.0 micrograms. Room-temperature was 26 -27°C. on both days of the experiment.

0.75 ml. of distilled water or standard solution was delivered from a microburette into each of several reaction tubes. About 1 ml. of day-old anthrone reagent (0.2% in 95% sulfuric acid) was then withdrawn from the supply in a 50 ml. burette and discarded in order to eliminate any of it which had absorbed moisture from the air. With an effort to reproduce the same rate of delivery each time and thus to minimize variable errors in measuring this relatively viscous liquid, 1.5 ml. of the reagent were

run down the insides of the reaction tubes from points close to the bulbs. Layering of the solutions occurred readily, and they were mixed abruptly by swirling as soon as each tube was filled. The tubes wei clipped in a holder, shielded from drafts, and allowed to cool to room-temperature, whereupon the contents were transferred into micro-tubes for the photometer and readings were obtained. As before, zero on the instrument was repeatedly checked against a clean tube of distilled water.
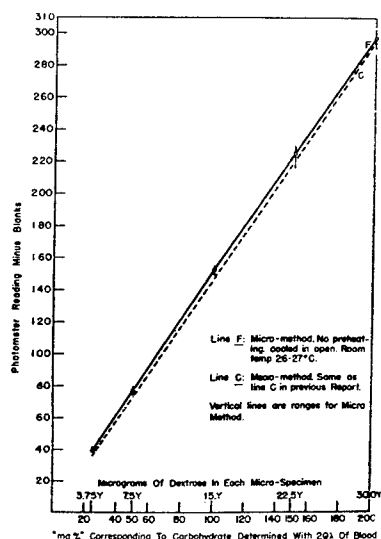
Results.

Figure 4. Averages and ranges for 10 series of paired determinations with standard solutions of dextrose and the micro-procedure (solid line).

In Fig. 4, line F, the averages of the photometer-readings-minus-blanks (ordinate) for ten series of duplicate micro-preparations are plotted against "mg.%" of dextrose corresponding to carbohydrate measured with 20 lambda of blood (abscissa, lower figures). However, for line C, which is the same as the one labeled C in Fig. 3 of Part I, "mg.%" and micrograms of dextrose are numerically identical (abscissa, lower figures). The similarity of color- development is evident in corresponding macro- and micro-specimens prepared in such ways as to give approximately the same relationship between amount of dextrose and final volume at the particular level of concentration of the sugar-solution being measured. What appears to be a constant difference between the results of the two methods could reasonably be attributed, at least in

part, to a fairly uniform and greater error in the measurement of the smaller quantities of the anthrone reagent. The vertical lines in Fig. 4 represent ranges for the combined readings of duplicate micro-specimens.

1. **General.** Line F shows that with the special reaction-tubes, the Bouguer-Beer law is obeyed for solutions of dextrose containing from 3.75 to 30 gamma in 0.75 ml. This fulfills a major requirement for the present objective of extending the use of anthrone in quantitative colorimetry. Furthermore, the agreement between results for large and small specimens, prepared with as many conditions and materials in common as practical, points to success in simulating on a micro scale the essential conditions governing color-development with a macro-technique of demonstrated usefulness. We have evidence indicating that the sort of approach described here would be successful with even lesser quantities if it were not for the limitation upon minimal volume imposed by our photometer.

Errors of measurement, which tend to be especially significant in connection with the anthrone reagent, can be expected to cause greater variability and absolute error with smaller amounts of material. Nevertheless, the results of this study offer promise for the fruitful clinical application of a micro-method using anthrone, at least when simplicity and convenience rather than utmost precision are desired. The very high degree of accuracy which Part I showed to be potentially obtainable from the color-developing reaction with dextrose might encourage further refinement without sacrifice of practical advantages.

2. **The control of temperature.** The assumption that insufficient heat is produced and retained with small quantities of material in ordinary test tubes, and a desire for simplicity, were the reasons for the special reaction tubes and the avoidance of less attractive methods of controlling temperature. However, it remains to be learned whether our compromise in favor of convenience is justified in the light of what is

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already known concerning differences in the behavior of various carbohydrates with the anthrone reagent (e.g., 3, 6).

We have found color-development to be reduced and more variable at low room-temperatures (e.g., below about 15°C. with the macro-procedure). In the case of the macro-method, this was corrected by insulating the tubes during cooling. The micro-procedure might require, in addition, some degree of preheating to compensate for cold solutions and the heat transferred to the reaction tubes. When not needed, however, preheating (to as little as 36°C. on a warm day) and the use of insulation result in departures from the Boguer-Beer law which increase with greater concentrations of sugar. In a warm room, specimens in the insulating box often show losses of color, after maxima have been reached, which can be followed visually.

Suggestions for a micro-procedure using blood.

It is not our intention to develop and evaluate a micro-method for blood, and our work with anthrone has ceased. However, the following suggested notes have been worked out in our laboratory:

Preparation of a series of specimens representing different standard concentrations of sugar should contribute to accuracy both through the weight of numbers and by revealing certain types of error not otherwise detectable. The less dilute sugars provide more sensitive indication of unsatisfactory control of temperature. Measurements for blanks are critical and also least accurate. It follows from Part I and from the lesser precision of a micro-procedure that duplicate blanks would not be sufficient, but the number of specimens which might be satisfactory for particular needs cannot yet be stated.

a. Sugar solution. Measurement and calculations are simplified by making the stock solution to contain 980 mg. of dextrose in a liter. Prepare sugar-standards from an intermediate 1:10 dilution of this stock by further dilutions in simple ratios: e.g., 1:20 for 25 mg.%; 1:10 for 50 mg.%, etc.

b. Blanks. Add 1.5 ml. of anthrone reagent (0.2% in 95% sulfuric acid), prepared the preceding day, to 0.75 ml. of 3-5% trichloroacetic acid in a reaction tube. Mix quickly and let it cool to room-temperature. Transfer to a Klett microtube and read in the photometer after adjusting the zero with a microtube of distilled water.

c. Standard sugars. Add 1.5 ml. of anthrone reagent to 0.75 ml. of sugar-solution ((a) above) in a reaction tube and continue as with blanks.

d. Blood-sugar. Add 20 cu. mm. of blood from a Sahli pipette to 1 ml. of 3-5% trichloroacetic acid in a centrifuge tube. Mix and spin at 3-4000 r.p.m. for 5-10 min. Place 0.75 ml. of supernatant in a reaction tube, and proceed as with blanks.

Notes. Filter paper and other extraneous carbohydrate sources must be scrupulously avoided. 3-5% trichloroacetic acid is used because, unlike several other deproteinizing agents, it gives a clear solution with the anthrone reagent. In the macro-procedure, blanks made with distilled water and with trichloroacetic acid are found to be equivalent if the acid is fresh and of good purity. The amount of liquid remaining behind on the walls of the Sahli pipette is less important than failure to approximate complete mixture of the sugar in the blood with the total amount of acid. Error, for which an empirical correction might ultimately be applied, can be expected to arise from the fact that the standard sugars and blood are measured differently. Our anthrone was purchased under the trade-name of "Microne" from the National Biochemical Co., 3106 W. Lake St., Chicago 12, Ill.

General Summary and Conclusions

An experiment was performed using a macro-procedure for the anthrone-hexose reaction with standard solutions of dextrose and a Klett-Summerson photoelectric colorimeter in order to reveal significant sources of error and variability and to assess the upper limits of accuracy of this reaction with reference to its suitability for

the clinical laboratory and other applications where precision may be required. Sensitivity and accuracy were found sufficient to permit the introduction of additional errors arising from the use of biological fluids without necessarily removing the anthrone-method from a position of advantage in competition with other procedures.

The eight items below pertain to work with a macro-method and Klett microtubes.

1. Slow fading of the anthrone-carbohydrate complex occured, which was equivalent to about 2 gamma for 25 gamma of dextrose and 7 gamma for 200 gamma of dextrose during 8 hours at 24°C.

2. Except for this fading, which is undetectable if specimens are read as soon as they have cooled, the Bouguer-Beer law was followed from 25-200 gamma of dextrose as closely as our measurements could indicate.

3. Such close agreement was obtained between duplicate preparations for each concentration of dextrose (means within 1 gamma of each other on Day 2) that it was not possible to differentiate variability caused by the anthrone-hexose reaction from that caused by the observers, the photometer, or preparations of the specimens.

4. Special care is required to reproduce equivalent volumes of the anthrone reagent in order to minimize measurable differences between preparations representing the same concentration of dextrose.

5. Careful observers may be used interchangeably to read the photometer without significantly increasing variability.

6. The variability of photometer-readings made by the same person is attributable to chance.

7. The smallest numbers of replications needed to justify expectations of certain percentages of error under idealized conditions are estimated for the blank and for four concentrations of dextrose.

8. It is considered possible that some microtubes may cause significant differences in photometer readings from those obtained with the majority of tubes for blanks and low concentrations of sugar.

The following items refer to a simple micro-technique, for which the preceding material helped provide a foundation:

1. When special reaction tubes designed to conserve heat were used in a warm room, the anthrone-hexose reaction obeyed the Bouguer-Beer law for dextrose in quantities of 3.75 to 30 gamma in 0.75 ml. of original solution.

2. Two supplementary methods of controlling temperature in order to obtain satisfactory color-development are considered, and effects of their improper use are mentioned.

3. A simple procedure is proposed for using Dreywood's anthrone reagent to measure "sugar" in 20 cu. mm. of blood collected in a Sahli pipette.

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